BIOLOGY OF REPRODUCTION **68,** 735–743 (2003)
Published online before print 17 October 2002.
DOI 10.1095/biolreprod.102.008433

Molecular Characterization and Expression of Porcine Bone Morphogenetic Protein Receptor-IB in the Uterus of Cyclic and Pregnant Gilts[1]

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ABSTRACT

Previous gene mapping analyses revealed a quantitative trait locus for uterine capacity on chromosome 8. Comparison of porcine and human genetic maps suggests that the bone morphogenetic protein receptor IB (BMPR-IB) gene may be located near this region. The objectives of this study were to 1) clone the full coding region for BMPR-IB, 2) examine BMPR-IB gene expression by the endometrium and its cellular localization in cyclic and pregnant gilts, and 3) map the BMPR-IB gene. By iterative screening of an expressed sequence tag library, we obtained a 3559-base pair cDNA clone including the full coding region of BMPR-IB. Endometrial BMPR-IB mRNA expression of White composite gilts was determined by Northern blotting in Days 10, 13, and 15 cyclic and Days 10, 13, 15, 20, 30, and 40 pregnant gilts. In cyclic gilts, endometrial BMPR-IB mRNA expression was elevated on Days 13 and 15 ($P < 0.01$) compared with Day 10. Expression of BMPR-IB mRNA was localized in both luminal and glandular epithelium on Day 15. However, in pregnant gilts, BMPR-IB mRNA expression was not significantly different in the endometrium from Day 10 to Day 20, and it was significantly decreased on Days 30 and 40 ($P = 0.011$). The BMPR-IB gene was mapped to 108 cM on chromosome 8. These findings show that BMPR-IB mRNA expression is regulated differently in cyclic and pregnant gilts; this pattern of gene expression may be important for endometrial function during the luteal phase of the estrous cycle as compared with early pregnancy.

female reproductive tract, gene regulation, kinases, polypeptide receptors, uterus

INTRODUCTION

Bone morphogenetic proteins (BMP) are members of the transforming growth factor (TGF)-β superfamily that play a pivotal role in bone formation during embryogenesis and fracture repair [1]. The biological effects of BMP are mediated by two specific subtypes of BMP receptors, known as BMPR-I and BMPR-II [2], that possess intrinsic serine/threonine kinase activity. Although type I and type II BMP receptors are able to bind ligands, signaling occurs via hetero-oligomeric complexes of BMP type I and type II receptors [3]. When ligand-BMPR complexes are formed, BMPR-II phosphorylates and activates the BMPR-I receptor, which triggers downstream events in the BMP signaling pathway [4]. Two subtypes of BMPR-I receptor, BMPR-IA and BMPR-IB (also known as ALK6), have been reported [5], and they are closely related structurally. However, they differ in their extracellular domains [6].

The expression of BMPR-IA, IB, and II mRNA in the granulosa cells and oocytes in ovaries of normally cycling rats [4] and throughout folliculogenesis in sheep [7] has been reported. Recently, Mulsant et al. [8], Souza et al. [9], and Wilson et al. [10] reported that the increased ovulation rate of the Booroola sheep with FecB phenotype was associated with a mutation in the kinase domain of the BMPR-IB gene. In the pig genome, quantitative trait loci (QTL) for ovulation rate were reported on chromosome 8 at approximately 105 cM by Rathje et al. [11], 29 cM by Wilkie et al. [12], and 5 cM by Rohrer et al. [13]. In addition, QTL for number of corpora lutea were reported on chromosome 8 at approximately 101 cM by Wilkie et al. [14] and 99 cM by Braunschweig et al. [15]. It is not known whether mutations in the BMPR-IB gene, which is predicted to be located on swine chromosome 8 by comparison with the human genome (see below), may occur in more prolific Chinese Meishan pigs and whether they influence ovulation rate similarly to mutations in sheep.

In addition to its role in ovarian physiology, the BMPRs have been implicated in the development of several organs during fetal life. In sheep, the weight of the fetal adrenal glands in homozygous carrier ewes of the BMPR-IB mutation is significantly less than in noncarrier (wild-type) ewes [9]. Booroola fetuses with FecB phenotype showed retarded development of the heart on Day 28, mesonephros from Day 30 to Day 40, and ovary from Day 30 to early neonatal life [16]. Furthermore, mutant mice lacking BMPR-IB had a thin uterine lining and an absent or underdeveloped endometrial gland [17], suggesting that BMPR-IB is needed for uterine development.

Uterine capacity is a component trait contributing to litter size in swine [18]. A QTL for uterine capacity was identified on the long arm of chromosome 8 near 71 cM [13]. The human BMPR-IB gene is located at the region of

[1]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.
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Received: 17 June 2002.
First decision: 6 July 2002.
Accepted: 28 August 2002.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Primers used in the characterization of the porcine BMPR-IB cDNAs and mapping of the gene.

Stage	Primer	Sequence
Partial clone with short 3′ UTR (pBMPR-IB1)	Forward	
	1070U	GGCTTCATTGCTGCAGACA
	1289U	CTGAAAAGTAAGAACATCCTGGTGAAG
	1979U	ATTCATCACCTCTGTTCG
	2261U	CTGTGACGCTGGAGAACAGG
	Reverse	
	1417L	ACTTCTGGAGCCATGTAGCGC
Full coding clone (pBMPR-IB2)	Forward	
	85U	CGCCTCTGAAGTGGATGTG
	599U	CACTGCCTCCGCTGAAGAC
	1372U	TACAAACGAGGTCGACATAC
	Reverse	
	510L	GGTGGCATTTACATCGCAAG
	1406L	CTGGGGTGTTGGGTGGTATG
	1417L	ACTTCTGGAGCCATGTAGCGC
	1766U	GCCAAGATGTCAGAGTCC
	2182L	CCGTCCAGTAGAGGTTCCCG
	2184L	CCGTCCAGTAGAGGTTCC
	3′-1-L	GAGCAGCCCTGGTATTTTGC
	3′-2-L	TCCCCTCCCTCCCTTAACTC
SNP discovery	BMPRIB-6F	AGCTGTGAAAGTGTTCTTCAC
	BMPRIB-7R	GTCCCTTTGATATCTGCA
SNP genotyping	Forward	GAAATGCAAATGGAACACCAG
	Reverse	CAGCCAGATCACCTCTAACTG
Probe		TTCTTAGGGATTTGAAGG

4q22–24 [19] or 4q23–24 [20], and comparison of the pig and human genetic maps indicates that this region shares homology with the uterine capacity QTL on porcine chromosome 8. Furthermore, the porcine epidermal growth factor (EGF) gene on chromosome 8 [21] is located within the previously reported uterine capacity QTL [13]. In sheep, BMPR-IB has been mapped to a region between the SPP1 and the EGF gene [22]. Thus, because of its known effect on ovulation rate, which may result in higher numbers of embryos in the uterus, early embryonic and fetal development, uterine development and its chromosomal location, we hypothesized that the BMPR-IB gene could affect endometrial function and thus early conceptus development and uterine capacity.

The full coding BMPR-IB cDNA sequences of mouse (GenBank accession number NM007560), ovine (GenBank accession number AF357007, AF312016, and AF298885), and human (GenBank accession number NM001203), along with the protein sequences of rat (GenBank accession number JC2491) and chicken (GenBank accession number Q05438), have been reported. However, the entire coding sequence of porcine BMPR-IB cDNA has not been reported. The objectives of this study were to clone the full-coding region for BMPR-IB, examine the changes and cellular localization of BMPR-IB mRNA expression in porcine endometrium, and map the BMPR-IB gene.

MATERIALS AND METHODS

Experimental Animals

Uterine endometrium from the middle of the right or left uterine horn was collected on Days 10, 13, and 15 from cyclic and Days 10, 13, 15, 20, 30, and 40 from pregnant White composite gilts (n = 3–4 each) for Northern blot analysis. A 1-cm uterine horn cross-section from the same region was collected on Days 9, 12, and 15 from cyclic and on Days 10, 13, 15, 20, and 40 from pregnant gilts (n = 3 each) for in situ hybridization analysis. All animal procedures were reviewed and approved by the U.S. Meat Animal Research Center Animal Care and Use Committee or by the Agricultural Animal Care and Use Committee, Texas A&M University (Animal Use Protocol 7-127).

Isolation of Porcine BMPR-IB cDNAs

Primer design was based on the conserved regions of the human (GenBank accession number NM001203) and mouse (GenBank accession number NM007560) BMPR-IB cDNA sequences. Forward (1071U) and reverse (1417L) primers (Table 1) were used to screen the ''Meat Animal Research Center (MARC) 2PIG'' (MARC 2PIG) expressed sequence tag (EST) library [23] by polymerase chain reaction (PCR). Iterative screening of the EST library revealed a clone (pBMPR-IB1) containing a part of the coding region and a short 3′ untranslated region (UTR). Further screening with a different forward primer (599U) and the same reverse primer (1417L), followed by a forward primer (85U) and a reverse primer (1417L), revealed a clone (pBMPR-IB2) containing the full coding region of BMPR-IB cDNA with a longer 3′ UTR. The two clones were sequenced in both directions by primer walking using vector (SP6 and T7) and specific primers (Table 1).

Northern blotting indicated that the size of one of the BMPR-IB transcripts is larger than the two clones obtained. Thus, the MARC 2PIG EST library was screened further with forward primers 85U or Sp6 and a reverse primer 510L by PCR to obtain clones containing a longer 5′ UTR. However, no clones with longer 5′ UTR were obtained. To search for a longer 3′ UTR, the MARC 2PIG EST library was screened further by PCR with a forward primer 2925U and a reverse primer 3′-1-L (Table 1) derived from pBMPR-IB2. This primer pair was designed to amplify all the plasmids containing 3′ UTR that were as long as or longer than p-BMPR-IB2. Then plasmid DNAs from positive wells were amplified by the same forward primer and T7 vector primer as reverse primer to select plasmids containing the longest 3′ UTR of BMPR-IB cDNA. A clone (pBMPR-IB3) containing a partial coding region and a longer 3′ UTR than pBMPR-IB2 was obtained. This clone was sequenced in both directions.

Northern Blotting

Northern blot analysis was performed using 30 μg of total RNA from endometrium. Total RNA was electrophoresed in 1.5% agarose gels prepared in 3-[N-morpholino] propane-sulfonic acid/formaldehyde buffer; the gels were then blotted onto Hybond-N nylon membranes (Amersham Life Science, Buckinghamshire, England). Probe for BMPR-IB was generated with T7 RNA polymerase using the MAXIscript kit (Ambion, Austin, TX) in the presence of [^{32}P]UTP and pBMPR-IB2 as the template. The membranes were prehybridized for 30 min using ULTRAhyb (Ambion) and hybridized with 1×10^6 cpm/ml radiolabeled probe at 68°C overnight. The membranes were washed once with 2× saline-sodium citrate (SSC), 0.1% SDS at 68°C and then with 0.1× SSC, 0.1% SDS at 68°C and subjected to autoradiography for 5 days. Later, the same membranes were

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P 248 ATCCAGAAGGTCGTATGCTTCGGTTAGCAGCAGCCTGTTTATCTGGTTCCAACTTCTGCTGAATCACAACCAGTTGTCCCTGAGCTATGACAAGAGAGGA 347
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O   1 atcaaggacgttctacactttggttatcagcagcctgtttatctggttcaaacttctgctgaatcacaagcattttccgttgagctatgacaagagagga 100

  348 AACAAAAAGTTAAAGGAGCAAGCCTGCCATACACCAGAAGCAAACTTCCTTGATAAC 404
       |||||||||||||  |||||||||| |||||   ||||||||||||||||||||||
  101 tacaaaaagttaaacaagcaagcctgtcatac.gtagaagcaaacttccttgataac 156
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FIG. 1. A comparison of the 5′ untranslated region for putative porcine (P) BMPR-IB cDNA with that of the reported ovine (O) sequence is shown. The sequence identity between the species was 86%.

stripped and hybridized with 18S RNA probe. Probe for 18S RNA was generated in the presence of [^{32}P]dCTP by PCR using a plasmid containing a partial 18S RNA gene as template and specific forward and reverse primers derived from the partial sequence of the porcine 18S RNA gene (GenBank accession number AF102857) to amplify 123 base pairs (bp). Membranes were prehybridized for 30 min in Rapidhyb (Ambion). Then 1×10^6 cpm/ml of radiolabeled probe was added and blots were hybridized at 65°C overnight. The membranes were washed with 2× SSC and 0.1% SDS at 65°C and then with 0.1× SSC and 0.1% SDS at 65°C and subjected to autoradiography for 10 min.

Statistical Analysis

Relative expression of BMPR-IB mRNA was determined by densitometry, and results were analyzed by ANOVA using the general linear models procedure of the Statistical Analysis System [24]. The model included effects of status, day of the cycle or pregnancy, and the status × day interaction. The day × status interaction was more fully evaluated using a set of orthogonal contrasts. Contrasts used to examine the effect of day within cyclic (C) gilts were 1) Day 13C versus Day 15C and 2) Days 13C and 15C combined versus Day 10C. Contrasts used to examine the effect of day within pregnant (P) gilts were 1) Day 10P versus Day 13P; 2) Days 10P and 13P combined versus Day 15P; 3) Days 10P, 13P, and 15P combined versus Day 20P; 4) Days 10P, 13P, 15P, and 20P combined versus Day 30P; and 5) Days 10P, 13P, 15P, 20P, and 30P combined versus Day 40P.

In Situ Hybridization Analysis

The localization of the BMPR-IB mRNA was determined in uterine sections by in situ hybridization analysis following a previously reported protocol [25]. At ovariohysterectomy, several sections (~0.5 cm) from the middle of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% (v/v) ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Then uterine tissue sections were deparaffinized in xylene and then rehydrated to water through a graded series of ethanol concentrations. Tissue sections were postfixed in 4% paraformaldehyde in PBS and then digested with Proteinase K (20 μg/ml) in digestion buffer (50 mM Tris, 5 mM EDTA, pH 8) for 8 min at 37°C. Sections were then refixed for 5 min in 4% paraformaldehyde, rinsed twice for 5 min each in PBS, dehydrated through a graded series of ethanol, and then dried at room temperature for 30 min. Sections were hybridized with radiolabeled antisense or sense cRNA probes generated from a linearized porcine p-BMPR-IB2 plasmid template (coding sequence 617–1878 bp) using in vitro transcription with [α-^{35}S]UTP (specific activity: 3000 Ci/mmol; Amersham). After digestion of the pBMPR-IB2 with restriction endonuclease XhoI, antisense cRNA probe was made by in vitro transcription using SP6 RNA polymerase and an SP6-specific primer. After digestion of pBMPR-IB2 with restriction endonuclease HindIII, sense cRNA probe was made using T7 RNA polymerase and primer. Radiolabeled cRNA probe (5×10^6 cpm/slide) was denatured in 75 μl hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl [pH 8], 5 mM EDTA [pH 8], 10 mM sodium phosphate [pH 8], 1× Denhardt, 10% dextran sulfate, 0.5 mg/ml yeast RNA, 100 mM dithiothreitol [DTT]) at 70°C for 10 min. Hybridization solution was applied to the middle of each slide and a coverslip was placed gently on top. Slides were then incubated in a humidified chamber containing 50% formamide/5× SSC and hybridized overnight at 55°C. Coverslips were removed by placing slides in 5× SSC/10 mM βME for 30 min at 55°C. Sections were then washed as follows: 50% formamide/2× SSC/50 mM βME for 20 min at 65°C; 1× TEN (0.05 M NaCl/10 mM Tris [pH 8]/5 M EDTA) for 10 min at room temperature; and then three times in 1× TEN for 10 min at 37°C. Sections were then digested with DNase-free RNase (10 μg/ml) in 1× TEN for 30 min at 37°C to remove nonspecifically bound probe and washed as follows: 1× TEN for 30 min at 37°C; 50% formamide/2× SSC/50 mM βME for 20 min at 65°C; 2× SSC for 15 min at room temperature; 0.1× SSC for 12 min at room temperature; 70% ethanol containing 0.3 M ammonium acetate for 5 min at room temperature; 95% ethanol containing 0.03 M ammonium acetate for 1 min at room temperature; twice in 100% ethanol; and three times in 1× TEN for 10 min at 37°C. Liquid film emulsion autoradiography was performed using Kodak NTB-2 liquid photographic emulsion (Eastman Kodak Co., Rochester, NY). Slides were stored at 4°C for 12 days, developed in Kodak D-19 developer (Eastman Kodak Co.), counterstained with Harris modified hematoxylin in acetic acid (Fisher, Fairlawn, NJ), dehydrated through a graded series of ethanol to xylene, coverslipped, and evaluated by both bright-field and dark-field microscopy using a Nikon Eclipse E1000 microscope (Nikon Instruments Inc., Melville, NY) and Act One software (Nikon).

Mapping

Primers to amplify across putative intron 6 were designed based on the human sequence. These primers correspond to bases 1088–1108 (forward) and 1210–1192 (reverse) as presented in Figure 1. The primer set amplified putative intron 6 and a portion of exons 6 and 7 of the porcine BMPR-IB gene. Based on the human genomic sequence, this intron was expected to be approximately 1.1 kb. Agarose gel electrophoresis of the resulting porcine genomic products indicated that the corresponding region of the pig gene was 0.9 kb. The amplified fragments from a subset of eight parents, comprised of one White composite boar and seven F1 sows [White composite × Meishan (3), White composite × Fengjing (1), White composite × Minzhu (1), or White composite × Duroc (2)] from the MARC Swine Reference Population [26], were bidirectionally sequenced and evaluated for polymorphisms. Chromatograms were imported into the MARC database, nucleotide bases were determined using Phred analysis [27, 28], and sequences were assembled into contigs using Phrap [29]. Polymorphisms were identified using Polyphred [30], interactively assessed using Consed [31], and tagged as accepted or rejected. Single nucleotide polymorphisms (SNPs) not detected by Polyphred were also tagged. After an SNP was detected, an assay was designed to score this polymorphism using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The primers used for the assay are indicated (Table 1).

RESULTS

Isolation and Characterization of Porcine BMPR-IB cDNA

Three cDNA clones, one containing the full coding region of porcine BMPR-IB (pBMPR-IB2; GenBank accession number AF432128) and two clones (pBMPR-IB1 and pBMPR-IB3) containing partial coding regions were isolated from the MARC 2PIG EST library. The pBMPR-IB2 cDNA consisted of 3559 bp with an open reading frame of 1506 bp that encoded 502 amino acids. It also contained 5′ and 3′ untranslated regions (UTR) of 404 and 1646 bp, respectively. One of the partial clones (pBMPR-IB1) matched pBMPR-IB2 starting from 1056 bp and contained a shorter (788 bp) 3′ UTR (GenBank accession number AF488733). The 3′ UTR matched the 3′ UTR of pBMPR-IB2 over its entire length until the polyA tail. Another partial clone (pBMPR-IB3) matched from 797 bp of pBMPR-IB2 but had a longer (3546 bp) 3′ UTR (GenBank accession number AF488734). The 3′ UTR of pBMPR-IB2 matched the 3′ UTR of pBMPR-IB3 until the polyA tail. The nucleotide sequence identity of the 5′ UTR of pBMPR-IB2 with that of the ovine BMPR-IB cDNA (GenBank accession number AF357007) was 86% (Fig. 1). The nucleotide sequence identity of the 3′ UTR of pBMPR-IB2 with

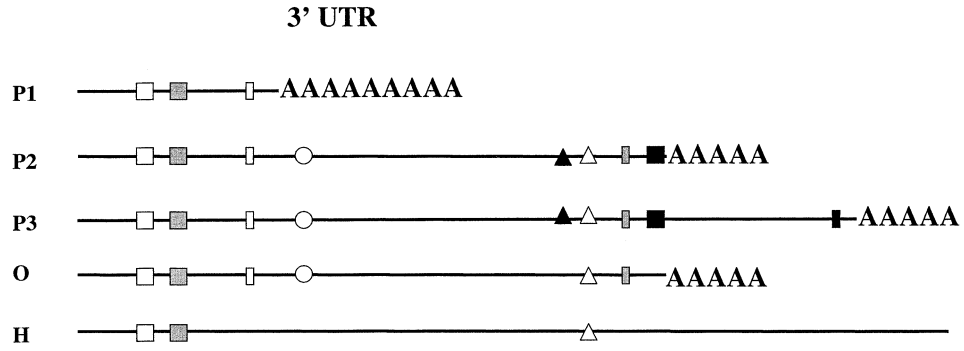

FIG. 2. A schematic diagram comparing the 3′ untranslated regions (UTR) of pBMPR-IB1 (P1), pBMPR-IB2 (P2), and pBMPR-IB3 (P3) with the 3′ UTR of the ovine (O) BMPR-IB cDNA sequence and the 3′ flanking sequence of the human BMPR-IB gene (H) is shown. The likely polyadenylation signal of AGTAAA sequences in pBMPR-IB2 and pBMPR-IB3 and a similar ACTAAA sequence in pBMPR-IB cDNA are indicated as gray rectangles. The alternative polyadenylation signals of AGTAAA, which are present in pMBPR-IB1, pBMPR-IB2, pBMPR-IB3, and ovine BMPR-IB cDNA, are indicated as white rectangles. The third alternative polyadenylation signal of ACCAAA sequence in pBMPR-IB3 is indicated as a black rectangle. The ATTTA sequence (white triangle) and the palindromic sequences GCTTTCTAAGAAAGC (white square) and ATTTTGCCAAAAT (gray square), which are conserved in three species, are indicated. The ATTTA sequence, present only in pBMPR-IB2 and pBMPR-IB3, is indicated as a black triangle. The palindromic sequence GGGAGTACCTCCC, which is only present in pBMPR-IB2 and pBMPR-IB3, is indicated as a black square. The CTTTTGATCTCTCAAATGAAAGGATC sequence, which is present in pBMPR-IB2 and pBMPR-IB3 and is conserved in the ovine but not in humans, is indicated as a circle.

that of the ovine BMPR-IB cDNA was 77% (Fig. 2). Two ATTTA sequences, a motif previously reported to destabilize mRNA [32], are located in the 3′ UTR of pBMPR-IB2 and pBMPR-IB3. One of these sites is fully conserved between porcine, ovine, and human sequences, while the other is not (Fig. 2).

Percent identities between the amino acid sequences of porcine BMPR-IB and ovine, mouse, and human BMPR-IB are 98.6%, 98.6%, and 98.2%, respectively. Potential N-glycosylation sites occur at amino acid numbers 284 and 344 and were conserved in ovine, human, and mouse sequences (Fig. 3). The transmembrane domains and the consensus ATP-binding sites were also conserved.

BMPR-IB Gene Expression

Two BMPR-IB mRNAs corresponding to 6.4 and 3.8 kb were expressed in the endometrium of gilts, and the 6.4-kb form was predominant (Fig. 4). Least-square means of densitometry units (± SEM) for BMPR-IB mRNA (the 6.4-kb form) in endometrium during the estrous cycle and pregnancy are illustrated (Fig. 4). A representative autoradiograph of a Northern blot of endometrial total RNA probed with [^{32}P]-labeled BMPR-IB cRNA and an autoradiograph of the same blot later probed with [^{32}P]-labeled 18S cDNA showing the matching 18S ribosomal RNA bands is illustrated (Fig. 4). There was a status × day interaction in

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  |→              extracellular domain
  1                                                  50 51                                                 100
P MLLRSSGKLN VGTKKEDGES TAPTPRPKIL RCKCHHHCPE DSVNNICSTD GYCFTMIEED DSGMPVVTSG CLGLEGSDFQ CRDTPIPHQR RSIECCTERN
M ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
O ---------S ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
H -----A---- ---------- --------V- ---------- ---------- ---------- ---L------ ---------- ---------- ----------

                          ←|
101                          transmembrane domain  150 151                                                 200
ECNKDLHPTL PPLKTRDFVD GPIHHKALLI SVTVCSLLLV LIILFCYFRY KRQETRPRYS IGLEQDETYI PPGESLRDLI EQSQSSGSGS GLPLLVQRTI
---------- ----D----- ---------- ---------- ---------- ----A----- ---------- ---------- ---------- ----------
---------- ----N----- ---------- ---------- ---------- ----A----- ---------- ---------- ---------- ----------
---------- ----N----- -----R---- ---------- ---------- ---------- ---------- ---------- ---------- ----------

     |→                                        kinase domain
201                                                 250 251                                                 300
AKQIQMVKQI GKGRYGEVWM GRWRGEKVAV KVFFTTEEAS WFRETEIYQT VLMRHENILG FIAADIKGTG SWTQLYLITD YHENGSLYDY LKSTTLDTKS
---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------A--
---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------A--

301                                                 350 351                                                 400
MLKLAYSSVS GLCHLHTEIF STQGKPAIAH RDLKSKNILV KKNGTCCIAD LGLAVKFISD TNEVDIPPNT RVGTKRYMAP EVLDESLNRN HFQSYIMADM
---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------P ----------
-------A-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------P ----------
---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------P ----------

                                                                                                        ←|
401                                                 450 451                                                 502
YSFGLILWEV ARRCVSGGIV EEYQLPYHDL VPSDPSYEDM REIVCIKKLR PSFPNRWSSD ECLRQMGKLM TECWAQNPAS RLTALRVKKT LAKMSESQDI KL
----------I ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --
---------- ---------- ---------- ---------- ---------- ---------- ---------- -----H---- ---------- ---------- --
---------- ---------- ---------- ---------- ---------- ---------- ---------- -----H---- ---------- ---------- --
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FIG. 3. A comparison of the predicted amino acid sequences for putative porcine (P) BMPR-IB with mouse (M), ovine (O), and human (H) sequences is shown. The beginning (|→) and the ending (←|) of the extracellular and kinase domains are indicated. The transmembrane domain is underlined and the consensus ATP-binding site is indicated by bold and underlining.

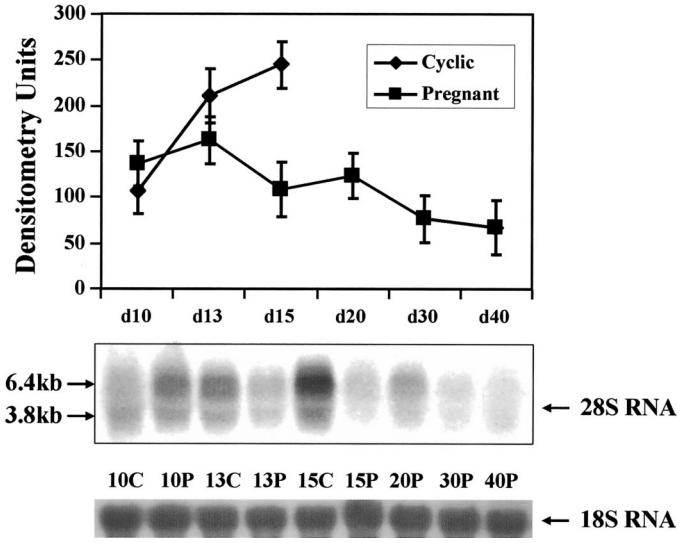

FIG. 4. Least-square means (± SEM) of densitometry units for BMPR-IB mRNA in endometrium during the estrous cycle and pregnancy are illustrated. In cyclic gilts (n = 3–4 animals each), BMPR-IB mRNA expression was elevated in the endometrium of Days 13 and 15 ($P < 0.01$) compared with Day 10. However, in pregnant gilts (n = 3–4 animals each), BMPR-IB mRNA expression did not change between Days 10 and 20 and was decreased on Days 30 and 40 ($P = 0.011$). A representative autoradiograph of a Northern blot hybridized with the BMPR-IB cRNA probe and later with 18S ribosomal RNA probe is shown. Gels were loaded with total cellular RNA (30 μg). Arrows indicate a BMPR-IB mRNA band and 18S and ribosomal RNA bands.

BMPR-IB mRNA expression ($P < 0.05$). In cyclic gilts (n = 3–4 each), BMPR-IB mRNA expression in the endometrium increased significantly from Day 10 to Day 13 and remained elevated on Day 15 ($P < 0.01$; Fig. 4). However, in pregnant gilts (n = 3–4 each), BMPR-IB mRNA expression was not significantly different in the endometrium between Days 10 and 20 and then decreased on Days 30 and 40 ($P = 0.011$; Fig. 4).

In Situ Hybridization Analysis

In situ hybridization analysis indicated that the pattern of BMPR-IB mRNA expression tends to be different for endometrium of cyclic and pregnant gilts (Fig. 5). In cyclic gilts, the BMPR-IB expression appears to be up-regulated on Days 12 and 15 compared with Day 9. In cyclic gilts, the majority of the BMPR-IB mRNA expression between Days 12 and 15 was localized to the luminal and deep glandular epithelial cells. In pregnant gilt endometrium, low levels of BMPR-IB expression were observed on Days 13, 15, and 20 in luminal epithelial cells. Low to no expression of BMPR-IB mRNA was detected in the endometrium on Day 40 of pregnancy.

Detection of a Point Mutation in Porcine Genomic DNA

Genomic DNA from endometrium of 10 White composite × Meishan gilts, which were derived from the MARC resource population, were amplified to determine whether the same point mutation of the BMPR-IB gene associated with the ovine Booroola phenotype was present in the population. Forward [8] and reverse [10] primers known to amplify the region harboring the mutation in the ovine BMPR-IB gene were used to amplify pig genomic DNA. For the reverse primer, a nucleotide of the reverse primer sequence was deliberately altered so that the same point mutation occurring in the pig as the Booroola sheep can be detected by the AvaII restriction digestion [10]. After PCR amplification, the products were treated with AvaII. Agarose gel electrophoresis showed only a single band, indicating that the point mutation did not occur in this group of pigs (Fig. 6).

Mapping of the BMPR-IB Gene

Relative genetic position of the BMPR-IB gene was determined using a C/T single nucleotide polymorphism (SNP) within intron 6. This SNP was selected for marker development to map the BMPR-IB gene based on the quality of the sequence data and the expected number of informative meiosis (i.e., the parent allele that could be tracked to the offspring) from the SNP discovery sequence data. Sequence data indicated that this base was heterozygous in three F1 sows and both White composite boars in the parental generation of the MARC swine reference population [26]. An assay for automated genotype scoring by MALDI-TOF mass spectrometry was developed based on the Sequenom (San Diego, CA) genotyping technology as described by Heaton et al. [33]. Both alleles were observed segregating within the reference population. Genotypic data were generated, and the marker resulted in 103 informative meioses. Genotype calls were parsed into the MARC genotype data base and the relationship between inheritance of the new marker and the inheritance of previously mapped microsatellite markers was analyzed (linkage analysis) using Cri-Map 2.4 software. The most likely map position of this marker was determined to be chromosome 8 at relative position 108 cM. The closest microsatellite markers in the region are SW194 and SW790 anchored on the current MARC swine chromosome 8 linkage map (http://www.marc.usda.gov/). The highest level of statistical support for linkage of BMPR-IB was with marker SW790, which generated a logarithm of odds ratio score of 21.64 with a 1% recombination rate. The relative genetic position of this gene is outside of the 95% confidence interval of QTL for uterine capacity [13].

DISCUSSION

This is the first report of the full coding region of porcine BMPR-IB cDNA. The amino acid sequences of BMPR-IB are highly conserved among mammalian species (>98%). In cyclic gilts, BMPR-IB mRNA expression appeared to be up-regulated in the endometrium on Days 13 and 15 ($P < 0.01$). Expression of BMPR-IB mRNA was localized to both luminal and glandular epithelium. However, in pregnant gilts, BMPR-IB mRNA expression was not significantly different in the endometrium between Days 10 and 20 and was significantly decreased on Days 30 and 40 ($P = 0.011$). These findings show that BMPR-IB mRNA expression temporally up-regulated on Days 13 and 15 of the estrous cycle and decreased as pregnancy progressed in gilts on Days 30 and 40. This pattern of gene expression may play a role in the changes in the endometrium that occur during the luteal phase of the estrous cycle as compared with early pregnancy.

The porcine 5′ UTR (404 bp) is larger than that of the reported ovine BMPR-IB cDNA (156 bp; GenBank accession number AF357007). The sequence identity between the reported 156 bp in the 5′ UTR of ovine BMPR-IB cDNA and the matching region of the 5′ UTR of porcine BMPR-IB cDNA was 86% (Fig. 1). The 273 bp of the 5′ UTR of the human BMPR-IB cDNA (GenBank accession number NM_001203) were 69% identical to those of the

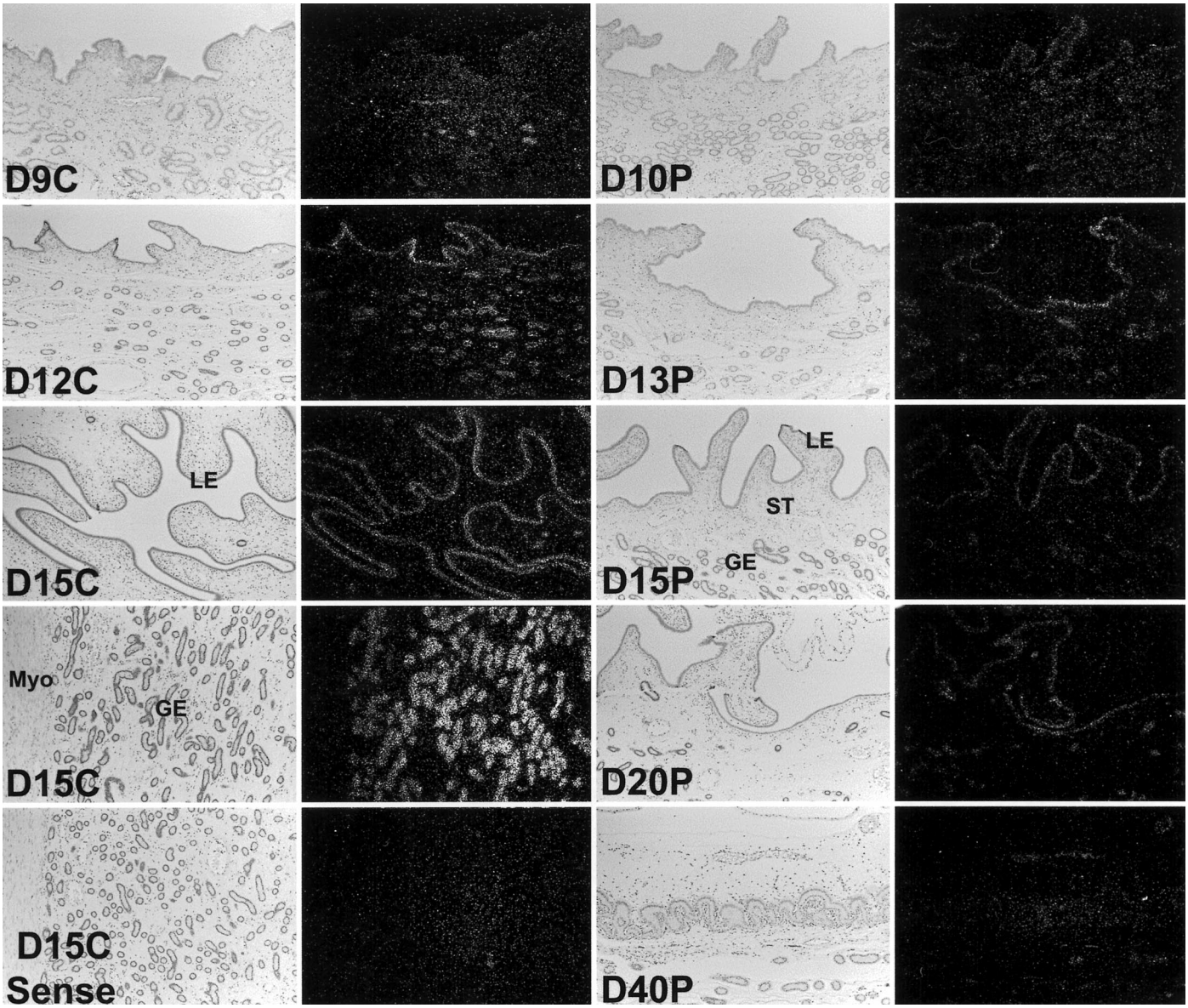

FIG. 5. In situ hybridization analysis of BMPR-IM mRNA in porcine endometrium from cyclic (C) and pregnant (P) gilts is illustrated. Corresponding bright-field and dark-field images of endometrium in cyclic (n = 3) and pregnant (n = 3) gilts are illustrated. A representative section hybridized with radiolabeled sense cRNA probe (D15C) serves as a negative control. Note that expression of BMPR-IB mRNA is limited to the LE and GE. LE, Luminal epithelium; GE, glandular epithelium; Myo, myometrium; ST, stroma. The width of each field is 13.2 μm.

porcine sequence (data not shown). Significant homology in the 5′ UTR of BMPR-IB cDNAs from different species suggests the possibility that this region may have some as yet undetermined functional significance.

The porcine 3′ UTR from the pBMPR-IB2 (1646 bp) is similar in size to that of the ovine BMPR-IB cDNA (1590 bp; AF357007). Sequence alignment between the porcine and ovine BMPR-IB cDNA showed conserved regions in the 3′ UTR including the second ATTTA sequence, an AGTAAA sequence, a 26-bp region, and two palindromic regions (Fig. 2). The 3′ UTR sequence of porcine BMPR-IB cDNA was 77% identical with that of ovine BMPR-IB cDNA. The 3′ UTR sequence reported for the human (250 bp; GenBank accession number NM_001203) and mouse (299 bp; GenBank accession number NM007560) are incomplete and do not contain polyA tails. In contrast, the reported ovine BMPR-IB cDNA (GenBank accession number AF357007) included a polyA tail, suggesting that its 3′ UTR sequence was complete. Because the short 3′ UTR (250 bp) of the human BMPR-IB cDNA may not be complete, the 3′ UTR of porcine BMPR-IB cDNA sequence was compared with the 3′ flanking sequence of the BMPR-IB gene from the human chromosome 4 sequence (GenBank accession number AC105395). This comparison revealed that the 3′ UTR from the pBMPR-IB2 is 73% identical to the 3′ flanking sequence of the human BMPR-IB gene (GenBank accession number AC105395). The extra 3′ UTR of pBMPR-IB3 was also 73% identical to the 3′ flanking sequence of the human BMPR-IB gene (GenBank accession number AC105395). The data suggest that a longer 3′ UTR of the BMPR-IB cDNA may exist at least in humans.

The AGTAAA sequence (3507 bp), 33 bp from the polyA tail of pBMPR-IB2, is likely to be the polyadenylation signal. The AGTAAA sequence (2618 bp), 18 bp from the polyA tail of pBMPR-IB1, is an alternative poly-

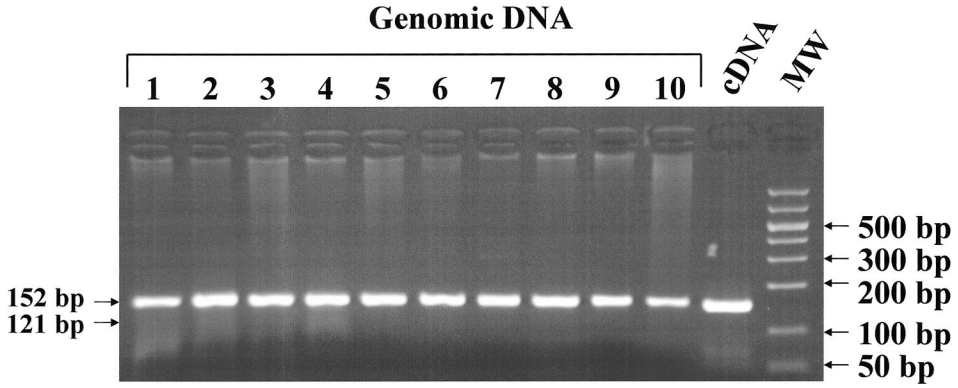

FIG. 6. Amplification of genomic DNA from 10 different pigs (lanes 1–10) and a BMPR-IB cDNA by PCR (lane 11), followed by AvaII digestion, revealed a 152-bp band. This suggests that no point mutation creating an AvaII site was present, which should generate two bands of 121 and 31 bp. Molecular weight (MW) markers of 50, 100, 200, 300, and 500 bp (lane 12) are indicated at right with arrows. The expected sizes of noncarrier (152 bp) and carrier (121 bp) of BMPR-IB mutation are indicated at left with arrows.

adenylation signal. The ACCAAA sequence, 45 bp from the polyA tail of pBMPR-IB3, is a third alternative polyadenylation signal. Thus, at least three alternative polyadenylation signal sequences for porcine BMPR-IB cDNAs appear to be present, resulting in at least three different forms of BMPR-IB cDNA. Sequence alignment between the porcine and ovine BMPR-IB cDNAs indicated that this AGTAAA sequence at 2618 bp of porcine BMPR-IB cDNA was conserved, suggesting that multiple alternative polyadenylation signals may be present in other species (Fig. 2). However, the presence of BMPR-IB cDNAs differing in their 3′ UTR has not been reported in other species.

When the 3′ UTR of the pBMPR-IB2 was compared with the 3′ flanking sequence of the BMPR-IB gene from the human chromosome 4 sequence (GenBank accession number AC105395), there was a conserved AGTAAA sequence near the polyA tail of the porcine BMPR-IB cDNA sequence. In the sheep, the ACTAAA sequence is present at the corresponding location, which would be the polyadenylation signal (31 bp from the polyA tail). Therefore, the three species may have similar sizes of 3′ UTR as pBMPR-IB2. The AGTAAA sequence at 2618 bp of the porcine BMPR-IB cDNA, which is likely the polyadenylation signal for pBMPR-IB1, is conserved in the ovine cDNA sequence, but AGTAAT is present in the human sequence. This suggests that one of the short forms of BMPR-IB mRNA may not be present in humans or another polyadenylation signal is used in humans. In humans, the AATAAA sequence is present approximately 80 bp downstream followed by a 32-bp polyT. This could be an alternative polyadenylation signal. Alternative polyadenylation signals resulting in different forms of 3′ UTR have been reported in other genes, including human estrogen sulfotransferase (GenBank accession number U55764 and Her et al. [34]), human fatty acid aldehyde dehydrogenase gene [35], and interferon-induced (2′–5′) oligo A synthetase [36]. Alternative polyadenylation could be a mechanism to regulate mRNA stability due to the presence of a sequence that may stabilize or destabilize mRNA in the longer forms of the 3′ UTR in such genes [32].

Two palindromic sequences in the 3′ UTR of pBMPR-IB1, pBMPR-IB2, and pBMPR-IB3 are conserved in the three species (Fig. 2). Signal scan analysis revealed that the ATTTTGCCAAAAT sequence is a binding site for transcription factor NF-1. Whether NF-1 is involved in the regulation of BMPR-IB or other genes in endometrium is yet to be determined. The importance of the other conserved palindromic region GCTTTCTAAGAAAGC is not known. The palindromic region GGGAGTACCTCCC of pBMPR-IB2 and pBMPR-IB3 is not present in either the ovine or human sequences, and therefore any possible functional significance of this sequence may be specific to pigs. The conserved CTTTTGATCTCTCAAATGAAAGGATC sequences of pBMPR-IB2 and pBMPR-IB3 are identical to the ovine sequence, but there are three mismatches in humans.

Two BMPR-IB mRNAs corresponding to 6.4 and 3.8 kb were expressed in the endometrium of gilts, and the 6.4-kb form was predominant. It is not known whether both 6.4- and 3.8-kb transcripts are translated and, if so, their translation efficiency. The intensities of the 6.4- and 3.8-kb bands tend to vary together, while the expression in mRNA as determined by densitometry was greater in 6.4-kb bands than 3.8-kb bands (data not shown). Therefore, analyzing the expression of mRNA using 6.4-kb bands emphasizes the changes occurring at different time points.

In humans, the 6.5-kb transcript was reported in the uterus and prostate, and the minor forms of 4- and 2.4-kb transcripts were reported in the prostate and testis [6]. In that report, the probe was generated using a cDNA fragment containing the 5′ UTR and the extracellular domain derived from the reported human BMPR-IB cDNA containing 2032 bp (GenBank accession number NM_001203). Sheep transcripts of approximately 6.2 kb were reported in adrenal gland, pituitary, kidney, and ovary [10]. In that report, a weak signal of 4.4 kb was also observed. Yet the longest of the ovine BMPR-IB cDNA reported previously contains 3255 bp (GenBank accession number AF357007). In this study, if we combine the 3546 bp of the long 3′ UTR from pBMPR-IB3 (GenBank accession number AF488734) with the 5′ UTR and coding sequence from the pBMPR-IB2 (GenBank accession number AF432128), the size becomes 5.5 kb. Still, that is smaller than the 6.4 kb determined by the Northern blot analysis. There may still be 5′ or 3′ UTR sequences yet to be characterized. Similar sizes of the transcripts in human (6.5 kb) and sheep (6.2 kb) further suggest that longer 5′ or 3′ UTR sequences that have not been reported in those species may also exist.

Our results are the first to show the presence of BMPR-IB mRNA in the endometrium of gilts and furthermore indicate that BMPR-IB expression is regulated differently in cyclic and pregnant gilts. The stage-specific patterns of BMPR-IB mRNA expression may be associated with changes that occur in the endometrium toward the end of the luteal phase of the estrous cycle. We speculate two possibilities based on other reports. First, Wasowska et al. [37] reported that, in pigs, the greatest number of apoptotic cells in the luminal and glandular epithelium was found on Days 17–19 and on Day 15 of the estrous cycle, respectively. Our in situ hybridization analysis and Northern blot analysis suggest that the elevated BMPR-IB may be involved

in the signaling of apoptosis in the endometrium. BMP-2 and BMP-4, which are the ligands for BMPR-IB, induce apoptosis in different human cell lines [38, 39]. Second, maintaining the basal level of BMPR-IB mRNA expression during early pregnancy may be needed to allow for the proliferation of endometrium, as mutant mice lacking BMPR-IB had a thin uterine lining and absent or underdeveloped endometrial glands [17]. The expression of the potential ligands for the BMPR-IB in the endometrium and endometrial glands in mice further supports this hypothesis [40, 41].

In conclusion, the full coding region for porcine BMPR-IB cDNA is reported. High nucleotide and amino acid sequence identity among the species suggests conserved function of BMPR-IB. The pattern of BMPR-IB gene expression by the endometrium suggests that it may play a role in modulating the uterine environment during the luteal phase of the estrous cycle as compared with early pregnancy. Alternative polyadenylation sites in the BMPR-IB gene are present and may be relevant to the control of either mRNA stability or translation efficiency. Finally, although confidence intervals overlap, the location of the BMPR-IB gene just outside the confidence interval for the uterine capacity QTL on chromosome 8 suggests that this gene may not be responsible for the QTL.

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